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Effects of Triclosan on Various Aquatic Organisms

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Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is widely used as an antibacterial agent in various industrial products, such as textile goods, soap, shampoo, liquid toothpaste and cosmetics, and often detected in wastewater effluent. However, there is a paucity of data on the toxicity of triclosan and its effects on aquatic organisms. In this study, the acute toxicity of triclosan to the Microtox® bacterium (*Vibrio fischeri*), a microalga (*Selenastrum capricornutum*), a crustacean (*Ceriodaphnia dubia*) and fish (*Danio rerio* and *Oryzias latipes*) was examined. As a result, the Microtox® bacterium, crustacean and fish had similar sensitivities towards triclosan toxicity (i.e., IC_{25} from 0.07 to 0.29 mg/L triclosan). In contrast, the microalga was about 30–80-fold (IC_{25} = 0.0034 mg/L triclosan) more sensitive to triclosan toxicity than the bacterium and fish. Therefore, triclosan is quite highly toxic to aquatic animals, and is particularly highly toxic to the green alga used as a test organism in this study. This result indicates that triclosan exerts a marked influence on algae, which are important organisms being the first-step producers in the ecosystem; therefore, the possible destruction of the balance of the ecosystem is expected if triclosan is discharged into the environment at high levels.

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1. Introduction

Various new chemical substances are produced with the advance of science and technology, and a considerable number of them are discharged to the aquatic environment. Triclosan (2,4,4'-trichloro-2'-hydroxy diphenyl ether of a chlorination diphenyl ether compound), which is widely used as antibacterial agent in various industrial products, such as textile goods, soap, shampoo, liquid toothpaste and cosmetics, is one of these substances. It is also used as a disinfectant in medical institutions because of its inhibiting effect on hepatitis B type virus.[1–4] Triclosan is detected in the drainage discharged following chlorine bleaching of textile.[5] It was reported that methylated triclosan was detected in fish and shellfish caught in Tokyo Bay and the Tama River.[6] In addition, triclosan can be transformed to 2,8-dichloro-dioxin easily by heating, which is in turn converted to dioxin upon sunshine irradiation.[7–9] Dioxin and dibenzofuran are detected in fly ash, indicating that triclosan is accumulated in dust incineration in the Netherlands,[10] which has great implications given the influence of dioxin on humans.[11] Therefore, it is necessary to clarify the mechanisms of dioxin production, such as processes that lead to the formation of precursors, and to prevent the discharge of dioxin to environments. However, there is a paucity of data on the toxicity of triclosan and its influences on aquatic organisms.

The purpose of this study was to determine the acute toxicity of triclosan to the Microtox® bacterium (*Vibrio fischeri*), a microalga (*Selenastrum capricornutum,* now *Pseudokirchnere subcapitata*), a crustacean (*Ceriodaphnia dubia*) and fish (zebrafish, *Danio rerio* and medaka, *Oryzias latipes*). The *C. dubia* reproduction test is widely used in aquatic toxicology. This test is known to be one of the most sensitive chronic toxicity tests and designed for regulating toxic materials in freshwater effluents by the U.S. Environmental Protection Agency (USEPA) and Environmental Canada. Zebrafish and medaka are relatively easy to breed in laboratory, and we can obtain their eggs all year round; hatching occurs approximately 2 and 9 days, respectively, after fertilization; the duration from fertilization to yolk absorption by the larvae is about 7 days.[12] The early life stage toxicity test using zebrafish and medaka is considered to be a sensitive biosensor for mammalian teratogenicity.[13,14] The algal test using *S. capricornutum* is required to evaluate toxicity to photosynthetic species and is standardized as well.[15–18] The Microtox® test is a simple, rapid and relatively inexpensive aquatic toxicity test that provides results that can be used as an indicator of toxicity measured by prescribed legal tests such as the acute lethality rainbow trout and daphnia assays.[19–23] We conducted an algal growth inhibition test, a water flea reproduction test, an early life stage toxicity test on two species of fish, and a Microtox® test. From the results of our tests we could clarify the toxicity of triclosan to the aquatic organisms tested.

2. Materials and Methods

2.1 *Test chemical*

Triclosan was purchased from Sigma Chemicals (St. Louis, MO, USA), and was dissolved with methanol.

2.2 *Biological test*

2.2.1 *Microtox® test*

Reagents, a freeze-dried bioluminescent bacterium *V. fisheri* (formerly known as Photobacterium phesphereum, NRRL number B-11177), and other required test solutions (dilution water, reconstruction buffer, osmotic adjusting solution) were purchased from Azur Environmental (Carlsbad, CA, U.S.A.). The experiment was carried out in accordance with the test conditions and operating protocol of the Microtox® acute toxicity test method.[24] Luminescence was measured with a Microtox® Model 500 photometer (Azur Environmental) in the acute mode. Various concentrations of triclosan solution (0.9, 0.45, 0.23, 0.11, 0.056, 0.028, 0.014, 0.007, 0.0035 and 0 mg/L) were prepared as test solutions in duplicate.

2.2.2 *Early life stage toxicity test using zebrafish and medaka*

Breeding conditions and maintenance procedures were carried out in accordance with those described by Piron.[14] Bloodstock fish were fed twice with Tetramin (Herrenteich, Germany) and once with live *Artemia* each day. After breeding, the fish were moved to stock aquaria, separating males from females. Eggs at the blastula stage were collected approximately 4 h after fertilization. They were rinsed to remove fecal matter and were transferred to 50-ml glass flasks containing 40 ml of triclosan solution. Various concentrations of triclosan solution (zebrafish; 0.5, 0.25, 0.125, 0.0625 and 0 mg/L, medaka; 1.25, 0.63, 0.31, 0.156, 0.0781 and 0 mg/L) were prepared in triplicate. Control flasks filled with water that was prepared by charcoal filtration were also used in each test. The test solution was provided with a sufficient amount of oxygen, and adjusted to pH 7.0±0.5 with NaOH or HCl. Water hardness was maintained at 200 mg/L $CaCO_3$. Dilution water (40 ml) was added to each flask, and 20 eggs were placed in each flask. During the test period, 80% of water in each flask was replaced every day. Live eggs were counted daily. The eggs were incubated in a climate chamber at 25±1°C and a 16-h light/8-h dark photoperiod. The fry were not fed during the test period, which was terminated more than 9 days (zebrafish) and 14 days (medaka) after hatching, when all of the fry had died.[25]

2.2.3 *Green algal growth inhibition test*

S. capricornutum Printz (NIES-35 strain) was obtained from axenic unialgal cultures in the National Institute for Environmental Studies (NIES), Tsukuba, Japan. Conical flasks (50 ml) covered with silicon caps (Shin-etsu Chemical Co., Ltd., Tokyo, Japan) were used for the culture and assay. They were shaken automatically at A00 rpm at 24±1°C under illumination at 4000 (±10%) lux. To obtain a preculture, a 0.1-ml aliquot of stock culture was inoculated into a 50-ml flask containing 20 ml of growth medium C,[26] and then incubated for 3 days. An equal amount (0.1 ml) of the precultured alga was added to a freshly prepared 20-ml medium C to obtain algal samples at the same growth phase. After 48-h incubation, 1 to 5 ml of the cultured alga was added to medium C. The final volume was adjusted to 20 ml, which contained 4×10^4 cells/ml. The flasks containing 20 ml each of test solutions at various triclosan concentrations were prepared for the assay. Triclosan solutions at ten concentrations (40, 20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0 μg/L) were prepared in triplicate. At the start of the assay, 1 ml of the precultured alga was inoculated into each flask.

After 72 h, cell density was measured using Coulter Counter ZM (Coulter Electronics Ltd., Fullerton, CA, U.S.A.). The rate of growth inhibition was calculated by dividing the number of cells in cultures containing triclosan at various concentrations by that in the untreated control culture. Comparison of regression lines was performed statistically according to the method of Sokal and Rohlf.[27]

2.2.4 *Ceriodaphnia dubia reproduction test*

C. dubia was cultured and maintained at 25 ± 1°C under a 16-h light/8-h dark photoperiod. The water used for *C. dubia* culture was a mixture of 33% Evian water (Calpis Co., Ltd., Tokyo, Japan) and 67% Volvic water (Mitsubishi Co., Tokyo, Japan) (v/v), which are commonly marketed as mineral water in Japan. This mixture was used in order to stabilize the water quality of test solutions, such as water hardness. *S. capricornutum* culture (0.3 ml; 4 × 104 cells/ml) and 0.3 ml of YCT (a mixture of yeast, Cerophyll, and trout chow used as food for *C. dubia*)[28,29] were added to each 400-ml culture beaker every day.

Survival and reproduction tests on *C. dubia* were continued for 7 days in accordance with the methods proposed by USEPA.[29] The percentage of living adults and the mean number of young produced by a female were calculated. Test medium with seven concentratic of triclosan (1, 0.5, 0.25, 0.125, 0.0625, 0.0313 and 0 mg/L) were prepared along with the diluents and control vehicle. Dilutions were made with fresh culture water. Ten replicate glass chambers (50 ml), containing one neonate born within 24 h, were used for each test concentration. These chambers were tightly closed with Teflon caps to prevent volatilization of test chemicals. The medium was renewed daily. Water quality was analyzed every day. Water hardness, pH and dissolved oxygen were 110 mg/L $CaCO_3$, 7.0 to 7.5, and 80% to 99%, respectively. Temperature was maintained at 24 ± 1°C. Testing was continued until 60% of the control animals had completed three broods (usually 6 to 7 days). For a test to be valid, we required more than 80% survival rate of control animals and ≥ 15 offspring per female over the 7-day test period.

2.3 *Statistical analyses*

Test results were analyzed statistically by hypothesis testing. The data were first tested for normality and homogeneity of variance. Dunnett's method[29–31] was employed to compare the treatment mean with the control mean to determine IC_{25} (the inhibiting concentration at which a reduction of 25% in survival or reproduction was observed), which was commonly used as the endpoint.[29] The curve fitting method was used to calculate IC_{25} and IC_{50} from the data. When the data were insufficient in terms of normality and homogeneity, Steel's Many-one Rank Test was used in the test for chronic toxicity to *C. dubia*.[29]

3. Results and Discussion

Triclosan is widely used as an antibacterial agent in industrial products and is discharged to the environment. There is, however, a paucity of acute and subacute toxicity data of triclosan to aquatic organisms.

The Microtox® test, a bacterial bioluminescence inhibition test, which was developed by

the Microbics Company (now Azur Environmental, Co., Ltd., Carlsbad, CA, U.S.A.), takes advantage of the phenomenon that toxins reduce the luminescence of luminescent marine bacteria. It was recommended in an USEPA-American Society for Testing and Materials (ASTM) document, and is used worldwide,[32] because it is easy to obtain consistent data with this test. In this study, we investigated the acute toxicity of triclosan using the Microtox® test. The results of the Microtox® test after a 15-min exposure are presented in Fig. 1 and Table 1. The IC_{25} value of triclosan was 0.07 mg/L. We also assessed the effects of triclosan on aquatic organisms, namely, a microalga (*S. capricornutum*), a crustacean (*C. dubia*) and fish (*D. rerio* and *O. latipes*). The results of the chronic toxicity tests are shown in Fig. 1 and Table 1. All results of the tests were plotted on a dose-response curve and the standard error was very small. The IC_{25} values of triclosan for *S. capricornutum*, *C. dubia*, *D. rerio* and *O. latipes* were 0.0034, 0.17, 0.16 and 0.29 mg/L, respectively. Orvos *et al.*[33]

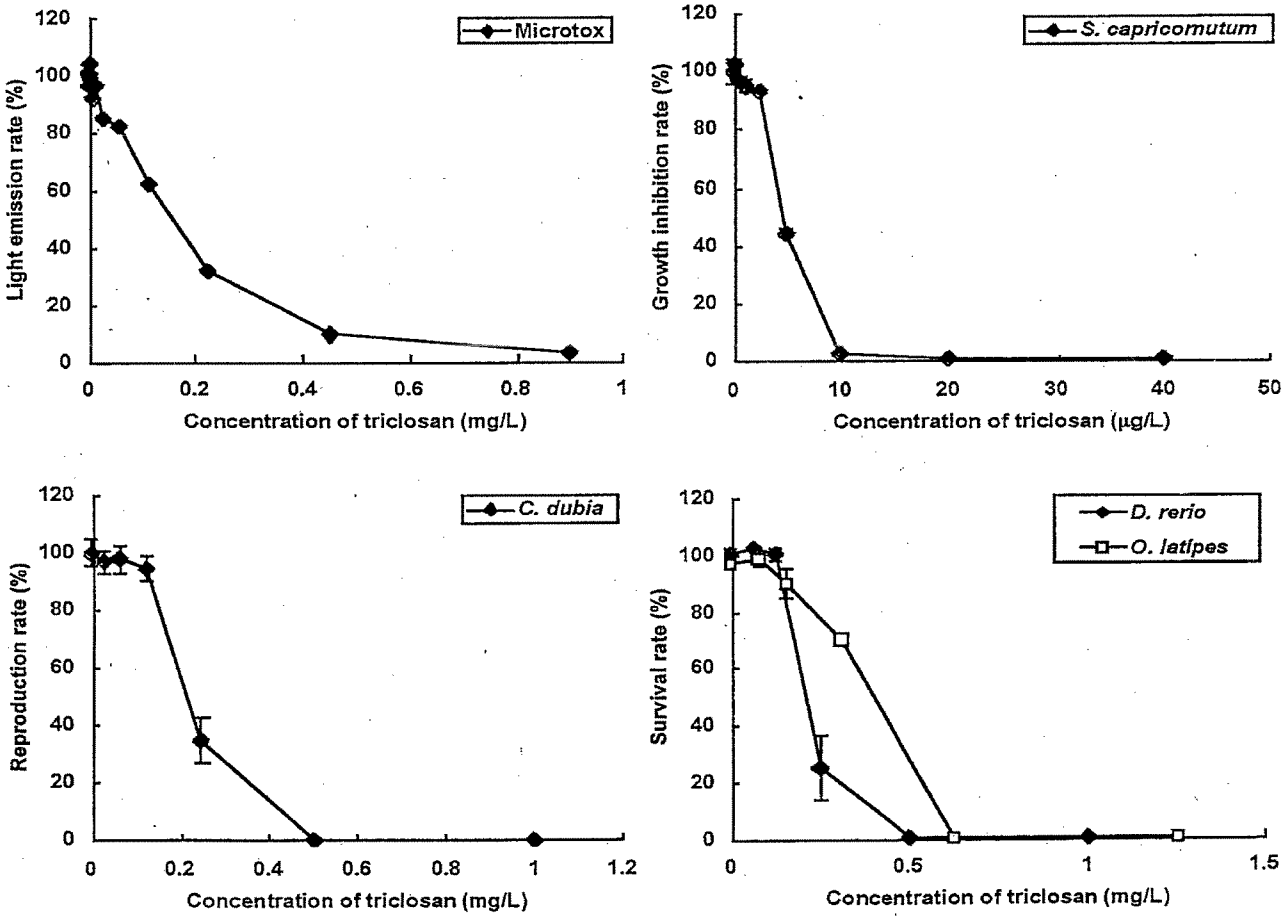

Fig. 1. Effects of triclosan on Microtox bacterium, growth of a green alga (*S. capricornutum*), reproduction of *C. dubia*, and survival of zebrafish (*D. rerio*) and medaka (*O. latipes*). Each value represents the mean ± S.E.

Table 1
Summary of IC_{25} and IC_{50} from the battery of five biological tests.

Test organism	IC_{25} (mg/L)	IC_{50} (mg/L)
Microtox	0.07	0.15
Selenastrum capricornutum	0.0034	0.0047
Ceriodaphnia dubia	0.17	0.22
Danio rerio	0.16	0.22
Oryzias latipes	0.29	0.40

studied the aquatic toxicity of triclosan using activated-sludge microorganisms, algae, invertebrates, and fish. The 48-h median effective concentration was 0.39 mg/L for *Daphnia magna* and the 96-h median lethal concentrations for *Pimephales promelas* and *Lepomis macrochirus* were 0.26 and 0.37 mg/L, respectively. Foran *et al.*[34] also reported that the 48-h LC_{50} for medaka fry was calculated to be 0.35 mg/L. Results of the present study are consistent with these results.

Our results showed that the bacterium, crustacean and fish had similar sensitivities towards triclosan toxicity in this study. In contrast, the microalga was 30–80-fold more sensitive to triclosan toxicity than the bacterium and fish. The sensitivity of the tested aquatic organisms to triclosan was in the order of green alga > bacterium > zebrafish > water flea > medaka. These results show that triclosan affects all the organisms (IC_{25}) at a concentration less than 0.3 mg/L. Therefore, triclosan is highly toxic to the aquatic animals, and is particularly highly toxic to green alga used as a test organism in this study. Triclosan is frequently found in wastewater effluent. Analyzed samples from Rhode Island were found to contain 10–20 μg/L triclosan in effluent and 80–100 μg/g triclosan in sediment near the outfall of a wastewater treatment plant.[35] Tanishima and Takada also detected 577 ng/L triclosan in wastewater treatment plants in Japan.[36] Moreover, the U.S. Geological Survey used five newly developed analytical methods to measure concentrations of 95 chemical compounds (such as pharmaceuticals, hormones, and other organic wastewater contaminants) in water samples from a network of 139 streams across 30 states from 1999 to 2000 to provide the first nationwide survey of the occurrence of organic wastewater contaminants in water resources.[37] In this study, triclosan was one of the most frequently detected compounds, and the concentration of triclosan exceeded the maximum allowable contamination level of 2.3 μg/L. These results indicate that triclosan exerts a significant influence on algae, which are important organisms being the first-step producers in the ecosystem; therefore, the possible destruction of the balance of the ecosystem is expected if triclosan is discharged into the environment at high levels. It is difficult to accurately evaluate the risk posed by triclosan to the environment, because many aspects of the dynamic state (the fate) of triclosan in the environment have not been clarified to date. However, we need to minimize the quantity of triclosan discharged to the environment, because this substance exerts influence on various organisms at a very small quantity.

References

1 Black, J.G. and Rutherford, T. (1975): Percutaneous absorption and metabolism of Irgasan DP300. *Toxicology* **3**: 33–47.

2 Kazama, S., Nakamura, Y. and Harada, H. (1980): Studies on analytical method of specific materials in cosmetics (X) high-performance liquid chromatographic analysis of germicides in cosmetic products. *Annual report of Tokyo Metropolitan Research Laboratory of Public Health* **31**: 91.

3 Yuge, O. (1983): The antimicrobial treatments for fabrics. *J. Antibact. Antifung. Agents* **11**: 76–81.

4 Amemiya, T., Sakai, M., Ikeda, K., Mori, K., Suzuki, S. and Watanabe, Y. (1985): Hygienic chemical studies on household necessities (XVI) dissolution test of Irgasan DP-300 and thiabendazole used as sanitary finishing agent in commercial textile products. *Annual report of Tokyo Metropolitan Research Laboratory of Public Health* **36**: 123–128.

5 Kanetoshi, A., Ogawa, H., Katsura, E., Kaneshima, H. and Miura, T. (1988b): Formation of polychlorinated dibenzo-p-dioxins upon combustion of commercial textile products containing 2,4,4'-trichloro-2'-hydroxydiphenyl ether (Irgasan DP300) and its chlorinated derivatives by exposure to sunlight. *J. Chromatogr.* **454**: 145–155.

6 Miyazaki, T., Yamagishi, T. and Matsumoto, M. (1984): Residues of 4-chloro-1-(2,4-dichlorophenoxy)-2-methoxybenzene (trimethyl) in aquatic biota. *Bull. Environ. Contam. Toxicol.* **32**: 227–232.

7 Pohland, A.E. and Yang, G.C. (1972): Preparation and characterization of chlorinated dibenzo-p-dioxins. *J. Agric. Food Chem.* **20**: 1093–1099.

8 Nilsson, C.A., Anderson, K., Rappe, C. and Westermark, S.O. (1974): Chromatographic evidence for the formations of chloro-dioxins from chloro-2-phenoxyphenols. *J. Chromatogr.* **96**: 137–147.

9 Kanetoshi, A., Katsura, E., Ogawa, H., Ohyama, T., Kaneshima, H. and Miura, T. (1992): Acute toxicity, percutaneous absorption and effects on hepatic mixed function oxidase activities of 2,4,4'-trichloro-2'-hydroxydiphenyl ether (Irgasan DP300) and its chlorinated derivatives. *Arch. Environ. Contam. Toxicol.* **23**: 91–98.

10 Olie, K., Vermeulen, P.L. and Hutzinger, O. (1977): Chlorodibenzo-p-dioxins and chlorodibenzofurans are trace components of fly ash and flue gas of some municipal incinerators in the Netherlands. *Chemosphere* **6**: 455–459.

11 Rappe, C., Nygren, M., Lidstrom, G. and Hansson, M. (1986): Dioxins and dibenzofurans in biological samples of European origin. *Chemosphere* **15**: 1635–1639.

12 Laale, H.W. (1977): The biology and use of zebrafish, *Brachydanio rerio* in fisheries research. A literature review. *J. Fish Biol.* **10**: 121–173.

13 ISO (1976): Final (revised) proposal for screening chemicals and products for acute toxicity to freshwater fish. ISO/TC 147/SC 5/wg 3 (Secretariat-6) 18. International Organization for Standardization.

14 Piron, R.D. (1978): Spontaneous skeletal deformities in the Zebra Danio (*Brachydanio rerio*) bred for fish toxicity tests. *J. Fish Biol.* **13**: 79–83.

15 OECD (1984): Guideline for the testing of chemicals: Algal growth inhibition 201. Organization for Economic Cooperation and Development.

16 ISO (1987): Water quality—Algal growth inhibition test. Draft International ISO/DIS 8692. Geneva, Switzerland. International Organization for Standardization.

17 US EPA (1989): Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. 2nd ed EPA/600/4-89/001 US EPA Cincinnati. OH. US Environmental Protection Agency.

18 Environmental Canada (1992): Biological test method: Growth inhibition test using the freshwater alga *Selenastrum capricornutum*. EPS 1/RM/25.
19 Dutka, B.J. and Kwan, K.K. (1981): Comparison of three microbial toxicity screening tests with the microtox® test. *Bull. Environ. Contam. Toxicol.* **27**: 753–757.
20 Bulich, A.A. (1982): The reliability of the bacterial luminescence assay for the determination of toxicity of pure compounds and complex effluents. *Aquat. Toxicity and Hazard Assess. ASTM STP* **737**: 338–347.
21 Peltier, W. and Weber, C. (1981): Comparison of the response of microtox®, fish and invertebrates to effluent toxicity. Aquatic Biology Section, Biological Methods Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio.
22 Goulden, C.E. (1982): Procedures and recommendations for the culture and use of daphnia in bioassay studies. *American Society for Testing and Materials, Special Technical Publication* **766**: 139–160.
23 De Zwart, D. and Slooff, W. (1983): The microtox® as alternative assay in the acute toxicity assessment of water pollutants. *Aquat. Toxicol.* **4**: 129–138.
24 Microbics Cooperation (1992): Microtox® acute toxicity test protocol.
25 Suter, G.W. and Rosen, A.E. (1987): Endpoints for responses of fish to chronic toxic exposures. *Environ. Toxicol. Chem.* **6**: 793–809.
26 Ichimura, T. (1983): Hybrid in viability and predominant survival of mating type minus progeny in laboratory crosses between closely related mating groups of *Closterium ehrenbergii*. *Evolution* **37**: 252–260.
27 Sokal, R.R. and Rohlf, F.J. (1981): Biometry-the principles and practice of statistics in biological research second edition: 826–837.
28 Cooney, J.D., DeGraeve, G.M., Moore, E.L., Palmer, W.D. and Pollock, T.L. (1988): Effects of food and water quality on culturing and toxicity testing of *Ceriodaphnia dubia*. Report EPRIEA-5820, Electric Power Research Institute, Palo Alto, CA.
29 US EPA (1994): Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 3rd ed. EPA/600/4-91/003. Office of Research and Development Cincinati. US Environmental Protection Agency.
30 Gulley, D.D., Boelter, A.M. and Bergman, H.L. (1989): 'TOXSTART Release 3.0'. University of Wyoming, Laramie, WY, USA, p. 18 + computer diskette.
31 Dunnett, C.W. and Tamhane, A.C. (1991): Step-down multiple tests for comparing treatments with a control in unbalanced one-way layouts. *Stat. Med.* **10**: 939–947.
32 Bulich, A.A. (1982): The reliability of the bacterial luminescence assay for the determination of toxicity of pure compounds and complex effluents. *In Aquatic Toxicity and Hazard Assessment: Fourth Conference ASTM STP 737* (Branson, D.R. and Dickson, K.L., Eds.), American Society for Testing and Materials, Philadelphia, pp. 338–347.
33 Orvos, D.R., Versteeg, D.J., Inauen, J., Capdevielle, M., Rothenstein, A. and Cunningham, V. (2002): Aquatic toxicity of triclosan. *Environ. Toxicol. Chem.* **21**: 1338–1349.
34 Foran, C.M., Bennett, E.R. and Benson, W.H. (2000): Developmental evaluation of a potential non-steroidal estrogen: triclosan. *Mar. Environ. Res.* **50**: 153–156.
35 Lopez-Avila, V. and Hites, R.A. (1980): Organic compounds in an industrial wastewater. Their transport into sediments. *Environ. Sci. Technol.* **14**: 1382–1390.
36 Tanishima, T. and Takada, H. (2003): Behavior of pharmaceutical chemicals in wastewater treatment plants. The 37th Japan Society on Water Environment Proceedings, Kumamoto, Japan: 348.
37 Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B. and Buxton, H.T. (2002): Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. *Environ. Sci. Technol.* **36**: 1202–1211.